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(54) Title: METHOD OF ASSAYING COMPOUNDS WHICH AFFECT THE ACTIVITY OF G PROTEIN-COUPLED RECEPTORS BASED ON MEASUREMENT OF RECEPTOR OLIGOMERIZATION

(57) **Abstract**

This invention is a new method of assaying compounds that modulate the activity of G protein-coupled receptors based on measurement of changes in the relative proportions of monomeric to multimeric receptor polypeptides. More specifically, techniques are described herein which permit the prediction of the pharmacological efficacy of drug candidates based solely on the ability of the candidate compounds to alter the ratio of receptor monomer to homo-oligomeric forms of the receptor. This method provides a novel means of assaying compounds as potential therapeutic drugs at G protein-coupled receptors which is greatly simplified and more generally applicable than existing methods.

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METHOD OF ASSAYING COMPOUNDS WHICH AFFECT THE ACTIVITY OF G PROTEIN-COUPLED RECEPTORS BASED ON MEASUREMENT OF RECEPTOR OLIGOMERIZATION

FIELD OF THE INVENTION

The present invention relates to a method of assaying compounds for the ability to modulate the function of G protein-coupled receptors based on measurements of receptor oligomerization state.

BACKGROUND OF THE INVENTION

The class of receptors known as G protein-coupled receptors (GPCRs) are typically characterized by a 7-helix organization, whereby the receptor protein is believed to traverse the membrane seven times. They also share a common signalling mechanism, whereby signal transduction across the membrane involves intracellular transducer elements known as G proteins. When a chemical messenger binds to a specific site on the extracellular surface of the receptor, the conformation of the receptor changes so that it can interact with and activate a G protein. This causes a molecule, guanosine diphosphate (GDP), that is bound to the surface of the G protein, to be replaced by another molecule, guanosine triphosphate (GTP), triggering another conformational change in the G protein. When GTP is bound to its surface, the G protein regulates the activity of an effector. These effectors include enzymes such as adenylyl cyclase and phospholipase C, channels that are specific for calcium ions (Ca^{2+}), potassium ions (K^{+}), or sodium ions (Na^{+}) and certain transport proteins.

In general, activation of GPCRs by transmitters will induce one or another of the following effector responses: activation of adenylyl cyclase, inhibition of adenylyl cyclase or stimulation of phospholipase C activity. When the effector adenylyl cyclase is either activated or inhibited it produces changes in the concentration of the molecule cyclic adenosine monophosphate (cAMP). Another effector, phospholipase C, causes one molecule of phosphatidylinositol-bisphosphate (PIP_2) to be cleaved into one molecule each of inositol triphosphate (IP_3) and diacylglycerol (DAG); IP_3 then causes calcium ions (Ca^{2+}) to be released into the cytoplasm.

Alterations in cellular levels of cAMP and Ca^{2+} are two of the most important intracellular messages that in turn act to alter the behaviour of other target proteins in the cell.

GPCRs may be classified according to the type of signalling pathway they activate in cells. This occurs at the level of the G proteins, which detect and direct signals from diverse receptors to the appropriate effector-response pathway. The three main groups of G proteins are: Gs-like, which mediate adenylyl cyclase activation; Gi-like, which mediate inhibition of adenylyl cyclase; and Gq-like, which mediate activation of phosphoplipase C. Since one receptor can activate many G proteins, the signal can be greatly amplified through this signal transduction pathway.

A wide variety of chemical messengers involved in regulating key functions in the body act through GPCRs. These include neurotransmitters such as dopamine, acetylcholine and serotonin, hormones of the endocrine system such as somatostatin, glucagon and adrenocorticotropin, lipid mediators such as prostaglandins and leukotrienes, and immunomodulatory proteins such as interleukin-8 and monocyte-chemoattractant polypeptide. The family of GPCRs also includes the receptors for light (rhodopsin), for odours (olfactory receptors) and for taste (gustatory receptors). Over one hundred different G protein-coupled receptors have been identified in humans, and many more are expected to be discovered. All or most of these receptors are believed to utilize one of the three principal G protein-effector signalling pathways (stimulation or inhibition of adenylyl cyclase or activation of phospholipase C).

Examples of G Protein-Coupled Neurotransmitter Receptors

Inhibits AC	Stimulates AC	Stimulates PLC	Neurotransmitter
m_2, m_4,		m_1, m_3, m_5	Acetylcholine
A_1, A_2			Adenosine
CRF-R			Corticotropin-Releasing Factor
R^c			Cannabinoids
D_2	D_1, D_5		Dopamine

	H_2		Histamine
Y_1, Y_2, Y_3			Neuropeptide Y
α_2-AR	β_2-AR	α_1-AR	Norepinephrine, epinephrine
μ, δ, κ	κ		Opioids
5-HT_{1A}, 5-HT_{1B}	5-HT_4	5-HT_2	Serotonin
5-HT_{1D}			

The development of a method of testing compounds for their abilities to affect GPCRs has great utility for many industries whose goal is to develop chemical compounds that interact with GPCRs. Since GPCRs are ubiquitous and widely used in nature to transmit cellular signals, this invention has utility for different industries including: the pharmaceutical industry, the pest-control industry, the aquaculture industry, the food industry and the fragrance industry.

In view of the diverse functions of G protein-coupled receptors in the human body, it is not surprising that the pharmaceutical sector has great interest in the development of new drugs which target G protein-coupled receptors for potential therapeutic applications in a wide range of human pathologies, including psychiatric disorders (depression, psychoses, bipolar disorder), metabolic disorders (diabetes, obesity, anorexia nervosa), cancer, autoimmune disorders, cardiovascular disorders, neurodegenerative disorders (Alzheimer's disease) and pain disorders.

The process of discovering and developing new therapeutic drugs which act on G protein-coupled receptors involves the systematic testing of drug candidate compounds in biological assay systems which contain the targeted G protein-coupled receptor in a functional state. The goal of this testing is to identify those compounds, among a very large number of candidates, which can modulate the function of the targeted G protein-coupled receptor in a predictable and therapeutically-relevant manner. Most assay systems used for drug screening classify compounds into three broad categories: 1) inactive, *i.e.* the compounds have no effect on receptor function at relevant doses; 2) agonists, *i.e.* the compound mimics the natural chemical messenger by activating the receptor; and 3) antagonists, *i.e.* the compound inhibits receptor

activation by the natural chemical messenger.

In general, two types of assay system are used by the pharmaceutical industry for screening compounds which target G protein-coupled receptors: ligand binding assays and functional bioassays.

The ligand binding assay detects compounds that can interact with and bind to the receptor at the same site as the natural chemical messenger. This usually involves the use of radioactive derivatives of either the natural chemical messenger or of known drugs which bind to the same receptor site, and measurement of the ability of test compounds to block the binding of the radioactive drug to the targeted receptor present in a biological preparation (e.g. a tissue extract). In addition to detecting compounds which bind to the receptor, the radioligand binding assay also permits the ranking of compounds based on binding affinity, i.e. the concentration of the compound which results in occupation of half of the receptors in the preparation. In general, the lower the concentration of compound necessary to occupy half of the sites (*i.e.* the higher the affinity), the better the candidate. Radioligand binding assays, while widely employed in the first steps of drug screening, have a number of limitations, the most severe being the inability of this assay to discriminate between agonists and antagonists.

The functional bioassay tests the effect of the compounds on receptor activity, *i.e.* the ability of the receptor to transmit signals across the cell membrane to control cellular response pathways. Since G protein-coupled receptors control a wide spectrum of cellular functions, the functional bioassays used in drug screening for G protein-coupled receptors include a large variety of different tests which monitor any one of a series of biochemical or cellular processes which are under the control of receptor activity. These assays all permit the discrimination of agonists from antagonists, i.e. agonists will activate receptor signalling pathways, while antagonists will block activation of signalling pathways by receptor agonists (such as the natural chemical transmitter). In addition, most functional bioassays can also rank agonist compounds based on efficacy, i.e. the maximum level of activation of the signalling pathway achieved by the agonist compound. Full agonists result in full activation of the receptor-controlled process, whereas partial agonists can induce only fractional activation of the

receptor-controlled process even at full receptor occupancy.

All of the functional bioassay systems currently used to screen drugs for GPCRs have a common element in that they all rely on the measurement of post-receptor processes as an indication of the direct effect of the compound on the receptor. Examples of functional bioassays performed to test the effect of compounds on the activity of G protein-coupled receptors cover the full spectrum of receptor-controlled processes including: (1) activation of G proteins in cell membrane preparations by measuring the rate of binding of the guanyl nucleotide analogue GTPγS35 or the hydrolysis of GTP; (2) modulation of effector activity as a result of G protein activation, *e.g.* activation or inhibition of adenylyl cyclase activity or activation of phospholipase activity; (3) modulation of post-effector signalling proteins, such as kinase and phosphatase enzymes, ion channels, transcription factors, *etc.*; (4) modulation of integrated cellular responses such as secretion (*e.g.* for glandular cells), contraction (*e.g.* for smooth muscle), electrical activity (*e.g.* for neurons), growth and proliferation (*e.g.* for endothelial cells).

All of these functional bioassays require the use of cells and/or cellular preparations that have a high degree of biological integrity. Until recently functional bioassays relied on the use of animal tissues. With the enormous progress in the cloning, sequencing and expression of genes which encode G protein-coupled receptors (and drug target proteins in general) there has been a major shift from the use of animal tissues to using recombinant receptors. Recombinant receptors are produced by expression of the cloned gene in cultured cells. In those cases where the receptor cDNA has been isolated and cloned such recombinant receptors are now the principal source of receptors for drug screening in ligand binding assays and in functional bioassays. The use of recombinant receptors has many advantages over tissue sources, including the ability to use human receptors expressed from human genes, the facility with which large amounts of the protein can be produced, and the fact that a single receptor subtype can be tested and compared against closely related subtypes (receptor subtypes are receptors that are closely related but distinct, yet which use the same natural transmitter).

Bioassay systems for recombinant G protein-coupled receptors that are known in the art are

based on the ability of the expressed receptor to activate endogenous signalling pathways in the host cell. Early assays measured the activity of effectors (adenylyl cyclase and phospholipase C) using known biochemical assays originally used for tissue-based assays. These generally employ mammalian cell lines which have been made to express the cloned receptor DNA using techniques (*eg.* transfection, transformation) which are well known to and routinely practised by technicians trained in the art. One example uses fluorescent dyes sensitive to the concentrations of specific ions, primarily calcium, to measure changes in the intracellular ion concentrations associated with activation of receptors coupled to Gq-phospholipase C signalling.

An increasing number of new assay systems involve genetic engineering of the host cell to facilitate measurement of the effector response to receptor activation. In gene reporter assays, the gene for an enzyme that is readily assayed, such as beta-galactosidase, is inserted into the host cell genome under the control of a gene promoter element which is normally under the control of a receptor signalling pathway. Receptors which activate the specific signalling pathway (will activate expression of the beta-galactosidase reporter gene. Measurement of the enzyme activity in a simple assay thus provides a measure of receptor activity and provides a functional bioassay to monitor the activity of compounds on the receptor. A variation of this type of assay uses the yeast *Saccharomyces cerevisiae* as a microbial host cell to express human G protein-coupled receptors which are coupled to an endogenous yeast signalling pathway controlling the response to sex pheromones. In this case, the receptor activates a yeast promoter which in turn controls the expression of a reporter enzyme (*e.g.* beta-galactosidase).

Another approach is to express the receptors in specialized cells that have endogenous response mechanisms that allow convenient assay of ligand activation of the receptor. For example, receptors that change cAMP levels have been cloned in melanophores (cultured pigment cells) wherein altered cAMP levels alter cellular colour, a response that is a conveniently measured response (Potenza *et al.*, 1992, Anal. Biochem. 206:315). The limitations of these types of assays are that only certain functional types of receptors can be measured, in addition to limitations in the endogenous responses and cells that are used.

Another assay strategy draws upon the observation that a wide diversity of receptors are able to alter the pH of the medium that is used for cell culture when exposed to ligands. These pH changes are small in magnitude and therefore require expensive instrumentation for measurement (Cytosensor, Molecular Dynamics Co.). Moreover, samples must be incubated within the instrument for several minutes which severely limits sample throughput.

Yet another assay strategy is based upon the ability of certain receptors to alter cellular growth. Cells of the NIH 3T3 fibroblast cell line have been extensively used to evaluate the activity of a large diversity of gene products that control cell growth, and a number of receptors are able to control the activity of these cells when stimulated by individual ligands. For example, carbachol (a muscarinic agonist) stimulates cells transfected with certain muscarinic receptors (Gutkind *et al.*, 1991, Proc. Natl. Acad. Sci. USA, 88:4703; Stephens *et al.*, 1993, Oncogene, 8:19), and Norepinephrine stimulates cells transfected with certain b-adrenergic receptors (Allen *et al.*, 1991, Proc. Natl. Acad. Sci. USA, 88:11354). In the course of long-term stimulation with agonist ligands, several characteristics of the cells are altered, including cellular growth, loss of contact inhibition, and formation of macroscopic colonies termed foci. Proprietary methods have been developed in the art in order to facilitate detection of such foci.

All of the functional bioassay systems used in screening share the common point that they measure post-receptor events, *i.e.* processes which are under the control of the receptor. This has several major drawbacks: (1) the measurement of compound efficacy is indirect and therefore subject to artifacts of the particular assay system used because post-receptor events may be modulated in unpredictable ways by unrelated cellular processes (*e.g.* other signalling pathways); (2) since the family of GPCRs is divided into functionally distinct groups, the particular functional bioassay will be limited in utility to a subset of the receptor family; (3) the functional bioassay systems based on post receptor events rely on complex biological systems which require a high degree of biological integrity to function effectively and they are therefore expensive and unstable.

Thus, there is great need for functional bioassays to assay compounds as potential drugs

affecting GPCRs which is simple, which is broadly applicable to functionally distinct receptors and which permits the direct measurement of compound efficacy on the receptor itself. Accordingly, it is an object of this invention to provide a functional bioassay method for assaying candidate drugs *in vitro* for their activity on GPCRs which meets these criteria. The assay system is based on the ability to monitor directly compound efficacy on GPCRs through a novel indicator of receptor activity: receptor oligomerization.

The background information is provided for the purpose of making known information believed by the applicant to be of possible relevance to the present invention. No admission is necessarily intended, nor should be construed, that any of the preceding information constitutes prior art against the present invention. Moreover, publications referred to in the following discussion are hereby incorporated by reference in their entireties in this application.

SUMMARY OF THE INVENTION

These objects are accomplished by the use a novel method of assaying compounds *in vitro* for their ability to interact with and modulate the functional properties of GPCRs. In particular, a techniques are described constituting a new method of testing compounds for their abilities to alter the relative amounts of monomeric and multimeric receptors. The invention is based on the discovery that GPCRs undergo reversible association-dissociation between monomeric and multimeric states (homo-multimers or hetero-multimers) as a normal part of their activity cycle, and that drugs affect this process, both in nature and extent, in a predictable manner. By using techniques generally known in the art to measure the relative amounts of monomeric and oligomeric receptors in a receptor preparation, the method of this invention permits direct measurement of the pharmacological efficacy of drug candidates at GPCRs.

TABLES AND FIGURES

While the specification concludes with claims particularly pointing out and distinctly claiming the subject matter regarded as forming the present invention, it is believed that the invention will be better understood from the following preferred embodiments of the invention taken in

connection with the accompanying drawings in which:

Figure 1 shows immunoblotting of human β_2AR expressed in Sf9 cells. Crude membrane preparations (lane 1), digitonin-solubilized membrane proteins (lane 2) and affinity purified receptors (lane 3) derived from Sf9 cells expressing either c-*myc* tagged (lane 3) or HA-tagged (lanes 1 and 2) β_2AR were immunoblotted following SDS-PAGE using the appropriate antibody (9E10 and 12CA5, respectively). The blots reveal immunoreactive bands corresponding to the expected monomeric form (43-50kDa) as well as a higher molecular weight species (85-95 kDa). The right panel illustrates immunoblots of crude membrane preparations derived from Sf9 cells expressing HA-tagged β_2AR treated (lane 5) or not (lane 4) with the membrane-permeant photoactivatible crosslinker BASED. Position of receptor bands are denoted by arrows and molecular weight markets are as shown.

Figure 2 shows effects of various peptides and β_2AR ligands on receptor dimerization. Co-immunoprecipitation of β_2ARs bearing two different immunological tags. Lanes 1 and 2: c-*myc* (lane 1) or anti-HA (lane 2) mAbs. The two immunoprecipitates were then immunoblotted with the anti-HA mAb. The occurrence of dimerization between the HA- and c-*myc*-tagged receptors is revealed by the fact that the HA-tagged β_2AR is co-immunoprecipitated with the c-*myc* tagged receptor by the anti-c-*myc* mAb (lane 1). Lanes 3 and 4: c-*myc* tagged β_2AR was expressed in Sf9 cells and immunoprecipitated with anti-c-*myc* mAb. The immunoprecipitates were then immunoblotted with either anti-HA (lane 3) or anti-c-*myc* or anti-c-*myc* (lane 4) mAbs. Lanes 5 and 6: HA-tagged β_2AR was expressed in Sf9 cells, immunoprecipitated with anti-HA mAb and then immunoblotted with either anti-c-*myc* (lane 5) or anti-HA (lane 6) mAbs. These controls demonstrate the specificity of each antibody towards their respective targets. Lane 7 and 8: HA-tagged β_2AR and c-*myc* tagged M2 muscarinic receptors were co-expressed in Sf9 cells, immunoprecipitated with either anti-HA (lane 7) or anti-c-*myc* (lane 8) mAbs. Immunoblotting with the anti-c-*myc* mAb did not reveal the presence of a β_2AR/M2 muscarinic receptor heterodimer (lane 8). Results shown are representative of three separate experiments.

Figure 3 demonstrates immunoblotting of V2-vasopressin receptors (V2-R) expressed in COS-

7 cells. Crude membrane preparations from COS-7 cells transiently transfected with c-*myc* tagged V2-R (lane 1) or c-*myc* tagged V2-R truncation mutant O-11 (lane 2) were immunoblotted with the anti-c-*myc* mAb. The molecular weight markets are as shown. Square brackets highlight the dimeric species of both wildtype and O-11 V2 vasopressin receptors while asterisks denote the monomeric species. Data are representative of three independent experiments.

Figure 4 shows effects of various peptides on receptor dimerization. A, Time course of the effect of the TM VI peptide on β_2AR dimerization. Membranes derived from Sf9 cells expressing β_2AR were treated at room temperature with TM VI peptide [residues 276-296: NH_2-GIIMGTFTLCWLPFFIVNIVH-COOH] at a concentration of 0.15 μg/μL for 0 (lane 1), 15 (lane 2), 20 (lane 3) or 30 minutes (lane 4). Membranes were then subjected to SDS-PAGE, transferred to nitrocellulose and immunoblotted with the anti-c-*myc* antibody. A representative immunoblot is shown. B, Densitometric analyses of three experiments similar to that shown in the Figure 4A demonstrating the effects of treatment for 30 minutes with either vehicle (CON, lane 1), TM VI peptide (TM VI, lane 2) TM VI-Ala [NH_2-AIIMATFTACWLPFFIVNIVH-COOH] (TM VI-Ala, lane 3), or D2 dopamine receptor TM VII peptide [residues 407-426 NH_2-YIIPNVASNVYGLWTFASYL-COOH) (D2 TM VII, lane 4). All peptides were used at a concentration of 0.15 μg/μL. The relative intensity of the dimer is expressed as percent of total receptor (monomer + dimer) immunoreactivity. Data shown are mean +/- SEM (n=3).

Figure 5 demonstrates, in **A**, effects of increasing concentrations of TM VI peptide on the amount of β_2AR dimer. Increasing concentrations (0-6.3 mM) of the peptide were added to purified c-*myc* tagged β_2AR and the amount of dimer assessed by immunoblotting using the anti c-*myc* mAb (lanes 1 - 8). In lanes 9 and 10 purified β_2AR was treated (lane 10) or not (lane 9) with the D2 TM VII peptide. The data shown are representative of three distinct experiments. Other control peptides used to determine the selectivity of the effect observed with the TM VI peptide included one derived from the C-terminal tail of the β_2AR [residues 347-358 NH_2-LKAYGNGYSSNG-COOH] or an additional control peptide unrelated to the β_2AR but of similar size as the TM VI peptide [NH_2-SIQHLSTGHDHDDVDVGEQQ-

COOH] were also found to be without effect on the amount of dimer (data not shown). **B**, Densitometric analyses of three experiments similar to that shown in B. The relative intensity of the dimer is expressed as percent of total receptor (monomer + dimer) immunoreactivity. *Inset* shows superimposed densitometric scans of immunoblotted receptors which were previously treated with increasing concentrations of the TM VI peptide. The monomer is denoted by M while the dimeric species is marked by D. The concentration of peptide added for the curves shown was: none (.....), 0.07 mM (---...----), 0.05 mM (--- ---), and 1.25 mM (------).

Figure 6 demonstrates effects of TM VI peptide on β_2AR stimulated adenylyl cyclase activity in Sf9 cells. **A**, Membrane preparations derived from β_2AR expressing Sf9 cells were either not treated (open circles), or treated with TM VI peptide (closed squares), control peptide TM VI Ala (closed circles), or second control peptide from TM VII of the D2 dopamine receptor (open triangles). Isoproterenol stimulated adenylyl cyclase activity was then assessed for these membranes. Data are expressed relative to the maximal stimulation obtained with the untreated membranes and represent mean +/- SEM for 8 independent experiments. Peptides were used at a concentration of 0.15 μg/μl. **B**, Effects of TM VI peptide (hatched bars) or vehicle alone (open bars) on basal (n = 13), maximal isoproterenol-stimulated (ISO, n = 13), forskolin-mediated (FSK, n = 13) and NaF-stimulated (n = 6) adenylyl cyclase activity was investigated. Data are expressed as pmol cAMP produced per mg membrane protein per minute +/- SEM. Statistical significance of the difference are indicated by an asterisk and represent a $p<0.05$ as assessed by a non-paired student's t-test. None of the control peptides discussed in figure 2 had effects on adenylyl cyclase stimulation in β_2AR expressing cells nor did any of the peptides have effects on adenylyl cyclase stimulation in Sf9 cells which were infected with the wildtype baculovirus (data not shown). **C**, Effects of increasing concentrations of peptide on isoproterenol and dopamine stimulated adenylyl cyclase activity were also investigated. Membranes were prepared from Sf9 cells expressing either the human β_2AR (open circles) or the human D1 dopamine receptor (closed circles). Adenylyl cyclase activity was measured using maximally stimulating concentrations of either isoproterenol (10^{-4} M) or dopamine (10^{-4} M) in the presence of peptide concentrations ranging from 10^{-8} to 10^{-4} M. Data were analyzed by non-linear least squares regression using SigmaPlot (Jandel

Scientific). The data are expressed as the mean +/- SEM (n = 3).

Figure 7 shows effects of β_2AR ligands on receptor dimerization. A, Time course of the effect of 1 μM isoproterenol on β_2AR dimerization. Membranes derived from Sf9 cells expressing the c-*myc* β_2AR were treated at room temperature with 1 μM isoproterenol for 0 (lane 1), 15 (lane 2), 20 (lane 3) or 30 minutes (lane 4). Membranes were then subjected to SDS-PAGE, transferred to nitrocellulose and immunoblotted with the anti-c-myc antibody. A representative immunoblot is shown. B, Densitometric analyses of three experiments where membranes from Sf9 cells expressing the β_2AR were treated for 30 minutes at room temperature with either vehicle (CON), 1 μM isoproterenol (ISO), 10 μM timolol (TIM), TM VI peptide at a concentration of 0.15 μg/μL (TM VI), or isoproterenol followed by 30 minutes with TM VI peptide (ISO/PEP). The TM VI data (lane 4) is reproduced from Figure 4b for comparison. The relative intensity of the dimer is expressed as percent of total receptor (monomer + dimer) immunoreactivity. Data shown are mean +/- SEM (n=3).

Figure 8 depicts effects of TM VI peptide on β_2AR expressed in mammalian cells. **A**, Effect of 0.15 ug/ul TM VI peptide (hatched bars) or vehicle (open bars) on basal (n=2), maximal isoproterenol-stimulated (ISO, n=2) forskolin-mediated (FSK, n=2) and NaF-stimulated (NaF, n=2) adenylyl cyclase activity in CHW cells expressing 5 pmol β_2AR/mg protein. Data are expressed as pmol cAMP produced per mg membrane protein per minute $\pm$ SEM. Statistical significance of the difference are indicated by an asterisk and represent a $p<0.05$ as assessed by a non-paired student's t-test. Membranes were treated with either vehicle (lane 1) or the TM VI peptide at a concentration of 0.15 ug/ul (lane 2) for 30 minutes at room temperature. Membranes from untransfected CHW cells had no detectable receptors (data not shown). **B**, Effects of TM VI peptide on β_2AR stimulated adenylyl cyclase activity in mouse Ltk- cells. Membranes were prepared from Ltk- cells stably expressing 200 fmol of human β_2AR/mg membrane protein. Isoproterenol-stimulated adenylyl cyclase activity was then assessed in membranes treated with vehicle (open circles), TM VI peptide (closed squares), control peptide TM VI Ala (closed circles), or the D2 TM VII control peptide (open triangles). Data are expressed relative to the maximal stimulation obtained with vehicle treated membranes and represent mean $\pm$ SEM for 3 independent experiments. Peptides were used

at a concentration of 0.15 ug/ul.

DETAILED DESCRIPTION OF THE INVENTION

The following common abbreviations are used throughout the specification and in the claims:

The abbreviation, IP is inositol phosphate.

The abbreviation BASED is bis [β-(4 azidosalicylamindo) ethyl] disulphide

The abbreviation, 5-HT is 5-hydroxytryptamine.

The abbreviation, DOI is 2,5-dimethoxy-4-iodoamphetamine hydrobromide.

The abbreviation, PBS is phosphate buffered saline.

The abbreviation, β_2AR is β_2-adrenergic receptor.

The abbreviation, GPCR is G protein-coupled receptor.

The abbreviation, GpA is glycophorin A.

The abbreviation, HA is influenza hemagglutinin.

The abbreviation TM VI is transmembrane domain 6.

The abbreviation, NDI is nephrogenic diabetes insipidus.

Orphan receptors are receptors for which the natural ligands and/or biological function are uncertain or unknown.

The present invention resides in the discovery that certain GPCRs form oligomeric structures (*eg.* homo-dimers) as part of their signalling activity, and that the effect of compounds on this process is predictive of the activity (agonist versus antagonist) and efficacy (partial and full agonist and inverse agonist) of the compound on the specific GPCR. A working example is provided, based on the human β_2 adrenergic receptor in which agonist promotes formation of oligomers, inverse agonist promotes dissociation of oligomers and a peptide derived from residues 276 - 296 of the β_2-adrenergic receptor inhibits agonist-promoted formation of oligomer and also inhibits stimulation of adenylyl cyclase activity. These results are completely unexpected for receptor aggregation has not previously been considered to influence or relate to activity of GPCRs.

This invention provides a method of testing compounds for activity and efficacy based on the

ability of the compound to alter the monomer-multimer equilibrium. This invention permits direct measurement of compound efficacy on the receptor independent of post-receptor signalling pathways, and is applicable to G protein-coupled receptors of different functional classes. Methods of screening compounds for activity and efficacy based on their ability to alter association-dissociation of GPCRs are unknown in the art because GPCRs are not thought to undergo association/dissociation as part of their activity.

It is surprising that measurements of multimer/monomer transitions in GPCRs would be reflective of activity, because it is not thought that GPCRs form multimers for activity; thus, testing for such a relationship is completely novel. The discoveries intimately relating receptor-multimer formation to receptor-activity gave rise to this invention. Thus, the idea of measuring multimer/monomer ratios instead of activities is an entirely new concept for a GPCR drug screening test.

This invention can also apply to orphan GPCRs, for which ligand specificity or receptor activities are not yet determined. The novel observations described in this invention indicate that receptors in the GPCR family will undergo this fundamental oligomerization process as an integral part of their activity, thereby allowing a novel method of assaying such receptors independent of knowledge regarding ligand specificity.

The method of this invention can also be used to test for compounds affecting the oligomerization of homo-multimers and hetero-multimers (comprised of polypeptides from different GPCR-types). For example it could be used to test for compounds that would affect the activity of hetero-multimers formed between 5HT-type receptor polypeptides and β-AR receptor polypeptides.

The techniques of this invention, measuring the multimer/monomer transitions in GPCRs, can either be correlated to receptor activity or used without such correlation. If they are so correlated for a particular type of GPCR, then assays can be conducted by determining the multimer/monomer ratio of the receptor in order to obtain an indication of its activity without

having to measure that activity directly, thereby obviating time consuming and costly procedures.

The principal goal of all the manifestations of the assay method embodied within this invention is to measure the category of action and the efficacy of drug candidates by determining these compounds' effects upon the ratio of monomeric receptor to oligomeric receptor (dimers, trimers, homo-multimeric, hetero-multimeric, *etc.*) The change in ratio of the relative amounts of monomer to multimer will reflect conversion of monomers to multimers or *vice versa*, thus providing information on the activity and efficacy of drug candidates. Those compounds which promote oligomerization would be predicted to have one activity (*eg.* agonist or positive efficacy) while those which promote dissociation of oligomers would be predicted to demonstrate the opposite activity (*eg.* inverse agonists or negative efficacy). The magnitude of change in ratio and/or rate of change effected by the compound would provide a measure of the compound's efficacy and/or potency in modulating receptor activity.

Measuring the ratio of monomeric receptor to multimeric receptor

There are many different techniques available for determining the relative amount of monomer to multimer (*eg.* dimer) formed in the presence and absence of the test compound. For example, different assay systems can be designed to measure the ability of compounds to modify the ratio of monomers/multimers. In general, any procedure that permits measurement of the relative amounts of monomer and oligomer in receptor preparations (*eg.* membranes, solubilized receptor preparations, purified receptors, *etc*) can be used. Typically, a sample containing the compound to be tested or a control sample lacking the compound would be added to a suspension or solution of receptor preparation. After an incubation period, the receptor preparation would be analyzed to determine the relative amounts of monomeric and oligomeric species such that changes in the ratio produced by the test compound could be used to predict the activity and efficacy of the compound in regulating receptor function.

Immunological methods can be used to measure compound efficacy. As demonstrated by the working example provided herein (see Figures and Examples) differential epitope tagging can

be used in combination with differential co-immunoprecipitation to demonstrate the formation or absence of multimeric subunit aggregation. As each type of subunit bears a unique tag, immunological techniques can be used to purify and identify the presence of each subunit in a multimer. If the complex is made up of two or more identical subunits (eg. homodimer or homotrimer), each subunit is treated as if it is unique, such that the subunits bear tags in proportion to the number of units in the multimer. For example, if the complex is a homodimer, one-half of the cDNA will be tagged with tag A and the other-half will be tagged with tag B. The resulting dimers will form between A-A, AB, and BB subunits, but will be observable by their migration in the SDS-PAGE gel, relative to the individual units. These will be visualized by immunoblotting with either or both types of anti-A MAbs or anti-B MAbs.

In a preferred embodiment of this invention, the following steps can be followed:

1) Synthesize sets of recombinant baculoviruses, wherein each set comprises cDNA encoding one subunit of a receptor and one unique immunologic tag, one set for each subunit;
2) co-express the sets of receptor cDNA, each set bearing a unique tag, in Sf9 cells;
3) solubilize membranes and purify receptors;
4) add test compound to the receptor preparation;
5) immunoprecipitate the receptors using anti-tag MAbs, one per unique tag;
6) separate the receptors using SDS PAGE;
7) immunoblot the SDS PAGE gel to observe resultant subunit aggregations;

An immunological method for measuring monomer/oligomer ratio entails separating monomers and oligomers based on size and measurement of relative amounts of each using reporter systems. In this embodiment the following steps would be followed:

1) receptor cDNA would be modified such that when expressed the expressed receptor would be tagged with the epitope for a monoclonal antibody: this expression would be performed in a heterologous system (eg. baculovirus-insect cell system);
2) the membrane preparation (or purified receptor) would be incubated with various

concentrations of compound for defined period;

3) membranes (or pure receptor) would be solubilized in SDS sample buffer and components separated by size on SDS-polacrylamide gels;

4) separated proteins would be transferred to nitrocellulose filter and relative positions of the tagged receptors visualized with anti-epitope antibody in an immunoblot reaction;

5) monomeric and oligomeric receptor species would be identified by size and relative amounts of each species determined by densitometric scanning;

6) the ratio of monomer/oligomer species would be compared for different concentrations of the test compound.

Using the techniques of this embodiment, alternate means of separating monomeric and oligomeric receptor species by size can be used: *eg*. gel filtration, ultracentrifugation or others followed by antibody detection of different size forms and determination of ratio of monomeric to oligomeric species. Alternate means of labelling the receptor could entail labelling the receptor with some reporter permitting specific detection of the receptor (*eg*. fluorescent label specifically incorporated into the receptor protein which can be quantified following size separation of monomeric and oligomeric species.

In yet a further embodiment, the association of monomers into oligomeric receptor complexes can be measured directly using Fluorescence Resonance Energy Transfer, involving use of two different fluorophores with distinct excitation and emission spectra, where the emission spectrum of the first fluor overlaps with excitation spectrum of the second fluor. Two separate preparations of receptor would be labelled with one or the other fluor and these labelled receptor preparations would be reconstituted together in solution or in phospholipid vesicles. The mixture would then be irradiated at the excitation wavelength of the first fluor. Monomers would show major emission and emission wavelength for the first fluor. Oligomers would show increased emission at the emission wavelength of the second fluor due to close proximity of the two fluors and energy transfer from the first to the second fluor. The ratio of emission intensities at the emission wavelengths for the first and second fluors would provide a measure of the relative amounts of monomeric (no energy transfer) and the oligomeric receptor species. Compounds which modify the ratio of monomeric and oligomeric

species of the receptor will also modify the ratio of emission intensities at the two emission wavelengths and permit prediction of activity and efficacy of the compound in regulating receptor activity.

Modifications to this Fluorescence Resonance Energy Transfer method can be made by using receptors tagged with different epitopes and two corresponding monoclonal antibodies labelled with first and second fluors. In this alternative method, two receptor populations (tag 1 and tag 2) in the same preparation (by co-expression of two receptors in insect cells or mammalian lines; or by separate expression and reconstitution into single preparation) are incubated with anti-tag 1 labelled with fluor 1, and anti-tag 2 labelled with fluor 2. Monomers will not show energy transfer between fluors 1 and 2 on different receptor monomers, whereas oligomers will bring two receptor-bound antibodies into proximity and permit energy transfer, measured as an increase in emission intensity at the emission wavelength of fluor-2. Compounds would be added to the mixture and tested for their abilities to promote receptor oligomerization or dissociation of oligomers into monomers, and this information would permit prediction of compound activity and efficacy in regulating receptor function.

The specifics of assessment assays for test compounds would thus involve the following steps: adding aqueous solution containing the test compound to be evaluated to solution containing a GPCR preparation (tissue, cell or extract); adding agonist to the same solution; measuring the response to agonist by means of an assay as described above; comparing the magnitude of the response to agonist in the presence of the peptide or peptidomimetic compound to that of the response in the absence of test molecule under otherwise identical conditions. Decrease in agonist-induced response in the presence of peptide or peptidomimetic compound indicates antagonist activity.

Activity of the test compound can be further characterized by testing: varying the concentrations of test compound against a fixed concentration of agonist to determine the potency of the antagonist-like test compound and then varying the concentration of the agonist with fixed test compound concentration to determine competitive versus non-competitive action. Finally, measuring the effect of test compound on progressively more distantly-related

receptors can be performed in order to determine selectivity.

Activity of the test compounds can also be assessed by measuring the compound's effect on spontaneous receptor activity (*i.e.*, basal activity in absence of added agonist). In this case, the same assay systems can be used but without agonist, and the decrease in receptor activity in presence of the test compound is measured.

EXAMPLES

Synthesis of peptides

Peptides were synthesized on solid-phase supports using f-moc chemistry (Merrifield, R.B., *Rec. Prog. Hormone Res.* 23:451-482, 1967; Stewart. J. and Young, J., *Solid Phase Peptide Synthesis*, Pierce Chemical Company, Rockford, Illinois, 1984) on a BioLynx 4175 manual peptide synthesizer (LKB). Peptides were solubilized in the following buffer: 100 mM NaCl, 10 mM Tris-HCl pH 7.4, 2 mM EDTA (plus a protease inhibitor cocktail consisting of 5 mg/ml leupeptin, 10 mg/ml benzamidine and 5mg/ml soybean trypsin inhibitor), 0.05% digitonin and 10% DMSO. Peptide sequences were confirmed by either mass spectrometry or amino acid analysis. Peptides used were as follows: (1) β_2AR TM VI peptide consisting of residues 276-296; NH_2-GIIMGTFTLCWLPFFIVNIVH-COOH, (2) a second peptide with Ala residues substituted at positions 276, 280, and 284; NH_2-AIIMATFTACWLPFFIVNIVH-COOH, (3) a peptide derived from residues 407-426 of the D2 dopamine receptor TM VII; NH_2-YIIPNVASNVYGLWTFASYL-COOH, (4) a peptide derived from the C-terminal tail of the β_2AR consisting of residues 347-358; NH_2-LKAYGNGYSSNG-COOH, and (5) an additional peptide unrelated to the β_2AR but of similar size as the TM VI peptide; NH_2-SIQHLSTGHDHDDVDVGEQQ-COOH.

Analysis of monomer/multimer ratios

To assess the effect of the different peptides on the β_2AR expressed in Sf9 and mammalian cells, the following experiments were performed. Generally, membrane preparations from

mammalian or Sf9 cells infected with recombinant baculovirus expressing human β_2AR were treated with increasing concentrations of the different peptides at room temperatures and for various times as indicated below. Specifically, membrane preparations from mammalian or Sf9 cells or affinity purified receptors derived from Sf9 cells expressing c-*myc* tagged β_2AR were treated at increasing concentrations of the different peptides at room temperature for various times as indicated (see results). Samples were then run on SDS-PAGE and then transferred to nitrocellulose. In some cases membrane preparations were also treated with either 10 μM timolol or 1 μM isoproterenol instead of, or in addition to the different peptides. Peptide antagonist activity was assessed by assaying adenylyl cyclase activity. In these assays, membranes were also used to determine the effect of various peptides on the ability of the β_2AR to stimulate adenylyl cyclase activity described below.

Recombinant baculoviruses

The recombinant baculoviruses encoding the c-*myc* or hemaglutinin (HA) tagged wildtype human β_2AR, the c-*myc* tagged human M_2 muscarinic receptor and c-*myc* tagged D_1 dopamine receptor (c-*myc* β_2AR and HA-β_2AR, c-*myc* M_2-R, and c-*myc* D_1-R respectively) were constructed as described (Mouillac, B., et al., J. Biol. Chem., 267:21733-21737, 1992). Briefly, HA (Tyr-Pro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala) and c-*myc* (Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu) tags containing initiator methionine residues were introduced into the receptor cDNAs immediately before their initiator methionines by subcloning the corresponding double-stranded oligonucleotides. Cells were infected with recombinant baculoviruses at multiplicities of infection ranging from 3-5.

Sf9 Cell Culture

Sf9 cells were maintained at 27°C in serum-supplemented [10% fetal bovine serum (FBS) v/v] Grace's insect medium (Gibco-BRL) with gentamycin and fungizone. Cells were grown either as monolayers in T flasks or in suspension in spinner bottles supplemented with pluronic acid to prevent cell taring due to agitation. Cells were infected at log phase

at a density of 1 x 10^6 cells per ml for 48 h.

Mammalian Cell Culture

CHW and LTK cell lines with and without stably transfected β_2AR were maintained as described (34). Cells were grown in Dulbecco's modified eagle medium (DMEM) supplemented with L-glutamate, 10% FBS, gentamycin and fungizone. Transfected CHW cells expressed 10^{-5} pmol receptor/mg protein while transfected LTK cells expressed 200 fmol receptor/mg protein. Stably transfected cell lines were grown in the presence of 150 μg/ml G418.

For transient expression of V2 vasopressin receptors the following procedures were followed. COS-7 cells were maintained in supplemented DMEM as described above. Genomic DNA for the V2 vasopressin receptor was isolated from nephrogenic diabetes insipidus (NDI) patients or unaffected individuals, subcloned into a construct containing a c-*myc* epitope tag and ligated into a mammalian expression vector, pBC12BI (Cullen, B.R, Meth. Enzymol., **152**:684-704, 1987). Using DEAE-dextran, COS-7 cells were transiently transfected with the expression vector encoding either wildtype V2 vasopressin receptor, a truncation mutant O-11 or with vector alone for 48 hours.

Membrane Preparation

Membranes were prepared as follows and washed. Sf9 or mammalian cells were washed twice with ice-cold PBS. The cells were then disrupted by homogenization with a polytron in 10 ml of ice-cold buffer containing 5 mM Tris-HCl, pH 7.4, 2 mM EDTA (plus a protease inhibitor cocktail consisting of 5 mg/ml leupeptin, 10 mg/ml benzamidine and 5 mg/ml soybean trypsin inhibitor). Lysates were centrifuged at 500 x *g* for 5 minutes at 4^0C, the pellets homogenized as before, spun again and the supernatants were pooled. The supernatant was then centrifuged at 45,000 x *g* for 20 minutes and the pellets washed twice in the same buffer. In some cases receptors were then solubilized in 2% digitonin or 0.3% N-dodecyl- -D-maltoside and purified by affinity chromatography on alprenolol-sepharose

as or by immunoprecipitation as described below.

Affinity purification of β_2ARs

Solubilized receptors were affinity purified by alprenolol-sepharose chromatography as described (Mouillac, B., et al., *J. Biol. Cem.*, **267**:21733-21737, 1992; Shorr, R.G.L., et al., *J. Biol. Chem.*, **256**:5820-5826, 1981). The affinity purified preparations were concentrated using Centriprep and Centricon cartridges (Amicon) and the amount of β_2AR in each sample was determined in soluble [^{125}I]CYP radioligand binding assays as described (Mouillac, et al., 1981, *supra*). Purified receptors were desalted on Sephadex G-50 columns prior to SDS-PAGE.

Immunoprecipitation of β_2ARs

Tagged β_2ARs were immunoprecipitated with either a mouse anti-c-*myc* monoclonal antibody (9E10; Evan, G.I., *et al.*, *Mol. Cell. Biol.*, 5:3610-3616, 1985) or a mouse anti-hemagglutinin monoclonal antibody (12CA5; Nimar, H.L., et al., *Proc. Natl. Acad. Sci. USA*, **80**:4949-4953, 1983) as described previously (Mouillac, et al., 1981, *supra*). Removal of digitonin and concentration of the solubilized receptor was performed by dialysis using Centriprep cartridges (Amicon) against an ice-cold solution (Buffer A) containing 100 mM NaCl, 10 mM Tris-HCl pH 7.4, 2 mM EDTA (plus protease inhibitors described above) until the digitonin concentration was reduced below 0.05%. Purified 9E10 or 12CA5 antibody (1:1000 dilution) was added to the concentrate and gently agitated for 2 hours at 4^{o}C. Anti-mouse IgG agarose (Sigma; at an 11:1 secondary to primary antibody molar ratio) and protease inhibitor cocktail were then added. The reaction was allowed to proceed overnight at 4^{o} C with gentle agitation. The immunoprecipitate was centrifuged at 12,000 rpm in a microcentrifuge for 10 minutes at 4^{o}C. The pellet was washed three times in buffer A and finally resuspended in 200 μL of non-reducing SDS PAGE loading buffer for 30 minutes, sonicated and centrifuged at 12,000 rpm. The supernatant was then subjected to SDS PAGE and Western blotting as described below.

Cross-linking of β_2ARs

Ten ml of Sf9 cell suspension (2 x 10^6 cells/ml) were taken 48 hours post-infection and either mock-treated with vehicle or treated with 1 mg of the membrane permeant photoactivatible cross-linking agent BASED (bis [β-(4 azidosalicylamindo) ethyl] disulphide; Pierce Chemicals) for 60 minutes at room temperature with gentle agitation. Membranes were then prepared from cells as described above and resuspended in non-reducing SDS PAGE sample buffer. Gels were subsequently immunoblotted as described below.

SDS-PAGE and Western blotting

Membrane preparations from Sf9 or mammalian cells or in some cases affinity-purified or immunoprecipitated β_2AR were prepared for non-reducing SDS-PAGE on 10% slab gels as described previously (Laemmli, U.K., *Nature*, 227:680-686, 1970). In the case of the V2 vasopressin receptors reducing SDS-PAGE was performed. For Western blotting, gels were transferred to nitrocellulose and blotted with either the mouse anti-c-*myc* monoclonal antibody (9E10), the anti-hemagglutinin monoclonal antibody (12CA5) at dilutions of 1:1000 or in the case of mammalian cells expressing the β_2AR, a polyclonal rabbit anti-β_2AR antiserum raised against a peptide from the C-terminal region of the β_2AR at a dilution of 1:2000. Immunoblots against the anti-c-myc or anti-HA antibodies were revealed using a goat anti-mouse alkaline phosphatase-coupled second antibody (GIBCO-BRL) or a chemiluminescent substrate for a horseradish peroxidase coupled second antibody (Renaissance, NEN Dupont). For the experiments performed using mammalian cells expressing the β_2AR western blots were developed using a chemiluminescent substrate for goat anti-rabbit coupled horseradish peroxidase antisera (Sigma). To assess total immunoreactivity of the various receptor species, blots were scanned by laser densitometry (Pharmacia-LKB Ultrascan).

Receptor quantification and adenylyl cyclase assay

Receptor number was calculated from saturation binding experiments using [^{125}I] cyanopindolol (CYP) as the radioligand (Bouvier et al., *Mol. Pharmacol.*, 267:7-19, 1994). Briefly, 10 μL of a membrane preparation in a total volume of 0.5 mL was labelled with 250 pmol of [^{125}I]-CYP which is at a near saturating concentration. Non-specific binding was defined using 10 μL alprenolol.

Adenylyl cyclase activity was assayed by the method of Salomon et al., (*Anal. Biochem.*, 58:541-548, 1974). Membranes were prepared and washed as described above. Again 10 μL of membranes (3-5 μg of protein) were used in a total volume of 50 μL. In some experiments, the peptides or the buffer used to solubilize them were added to the enzyme assay mix. Enzyme activities were determined in the presence of 1 nM to 100 μM isoproterenol, 100 μM forskolin or 10 mM NaF. Data were calculated as pmoles cAMP produced/min/mg protein and were analyzed by least squares regression using SigmaPlot 4.17 (Jandel Scientific).

Analysis of results

Immunoblotting of c-*myc* epitope tagged β_2AR expressed in Sf9 cells with the anti-c-*myc* antibody consistently revealed the presence of molecular species corresponding to the anticipated monomeric receptor (43-50 kDa) in Sf9 cells (Mouillac, et al., 1981, *supra*) as well as higher molecular weight forms. In particular, a prominent band was detected at an apparent molecular weight corresponding to twice that of the monomer (85-95 kDa) suggesting the existence of an SDS-resistant dimeric species of the receptor. In some membrane preparations discrete bands which could represent even higher order structures of the β_2AR can also be detected (Figure 1, lane 1). The dimer, which was readily observed in membrane preparations, was also detected in digitonin-solubilized receptors (lane 2) and following affinity purification of receptors on alprenolol-sepharose (lane 3). As shown in lanes 4 and 5, when whole cells expressing the β_2AR were treated with the membrane permeant cross-linking agent BASED, the dimer to monomer ratio as assessed by immunoblotting was increased by two-fold. This suggests that the dimer is already present before cell fractionation and that crosslinking stabilizes this form of the receptor.

therefore, the dimeric species does not represent an artifact of membrane preparation or solubilization. Identical results were obtained when membranes were solubilized with 0.3% N-dodecyl-β-D-maltoside instead of digitonin (data not shown)

In order to demonstrate that the higher molecular weight species observed in this study corresponded to a specific β_2AR homodimer, we devised a differential co-immunoprecipitation strategy using c-*myc* and hemagglutinin (HA) epitope tagging. Human β_2ARs bearing either of these tags were co-expressed in Sf9 cells. The receptors were then immunoprecipitated with the anti-HA or anti-c-*myc* antibodies, subjected to SDS PAGE and blotted with one or the other antibody. In the results shown in Figure 2 the anti-HA mAb was used to blot receptors immunoprecipitated with either the anti-HA mAb or the anti-c-*myc* mAb. As seen in lane 2, blotting of the anti-HA immunoprecipitate revealed both the 45 kDa and the 90 kDa forms of the receptor. The β_2AR could also be detected by the anti-HA mAb in the c-*myc* immunoprecipitate of co-expressed receptors but the dimer then represented the predominant form (lane 1). This indicates that the two molecular species (HA-tagged and c-*myc*-tagged β_2ARs) were co-immunoprecipitated as part of a complex which is stable in SDS, consistent with the higher molecular weight form being a β_2AR homodimer. Similar but complementary results are obtained when co-expressed receptors are immunoprecipitated with either anti-c-*myc* or anti-HA antibodies and then immunoblotted with the anti-c-*myc* or anti-HA antibodies and then immunoblotted with the anti-c-*myc* antibody (data not shown). The specificity of the mAbs is illustrated by the absence of cross-reactivity in cells expressing one tagged receptor species only (Figure 2 lanes 3-6). The occurrence of intermolecular interactions appears to be receptor-specific. Indeed, although dimers of c-*myc* tagged M_2 muscarinic receptor could be detected in Sf9-derived membranes expressing this receptor (data not shown and see Debburman, S.K., et al., *Mol. Pharmacol.*, 47:224-233, 1995) no co-immunoprecipitation with the HA-tagged β_2AR was detected when the two receptors were co-expressed (Figure 2, lanes 7,8).

<u>V2 vasopressin receptors are also dimeric.</u>

The vasopressin receptor is critical for regulation of water retention in the kidney. Recently, several mutations of this receptor have been linked to congenital nephrogenic diabetes insipidus (NDI, Bichet, D.G., et al., *Am J. Hum. Genet.*, 55:278-286, 1994). In another approach to demonstrate GPCR dimer formation, transient expression of both wildtype and a truncated form of the V2 vasopressin receptor in COS-7 cells was studied. Both monomeric (appx. 64-69 kDa) and dimeric (appx. 120-135 kDa) forms of the wildtype human V2 vasopressin receptor were detected when expressed in COS-7 cells (Figure 3, lane 1). A mutant form of the V2 receptor truncated in the C-terminal tail at residue 33y (O11, isolated from a patient with congenital nephrogenic diabetes insipidus (Bichet, D.G. et al., *supra,* 1994) was also capable of forming dimers when expressed in COS-7 cells (Figure 3, lane 2). Indeed, the O11 V2 receptor was detected as approx. 55-58 kD and appx. 89-100 kDa species consistent with the idea that higher molecular weight form represents a homodimer. These results confirm by a different approach that G protein-coupled receptors can form SDS-resistant dimers when expressed in mammalian cells.

Modulation of β_2AR dimerization by TM VI peptide

As shown in Figure 4a the addition of the TM VI peptide substantially reduced the amount of β_2AR dimer detected in Sf9 membranes in a time-dependent fashion (Figure 4a, lanes 1-4). In this experiment the relative amount of receptor dimer was gradually reduced from 54% at time zero to 17% after 30 minutes of treatment with TM VI peptide. When results of three such experiments were averaged, the TM VI peptide was found to reduce the relative amount of dimer by 69% after 30 minutes (Figure 4b). A control hydrophobic peptide (from transmembrane domain VII from the D2 dopamine receptor) at maximal concentration had no effect on the relative amount of dimer detected. (Figure 4b). This does not appear to result from a non-specific hydrophobic interaction since the unrelated dopamine receptor TM VII peptide was without effect. To address the importance of the glycine and leucine residues identified above, a second control peptide corresponding to TM VI of the β_2AR with Gly 276, Gly 280 and Leu 284 replaced by alanine residues (TM VI Ala) was synthesized. Although this peptide slightly decreased the amount of dimer its

effect was very modest compared with that of the TM VI peptide (Figure 4b) thus suggesting that these three residues may be a part of the interface between two receptor monomers. One mechanism which could explain the effect of the TM VI peptide is that it may interact with monomeric β_2AR thus preventing it from interacting with a second receptor monomer.

The effect of the TM VI peptide on dimer formation was also detected using purified β_2AR preparations and was shown to be dose-dependent. As seen in Figure 5a, increasing concentrations of the TM VI peptide led to a gradual reduction in the amount of dimer. This was accompanied by a concomitant increase in the level of the monomer such that the proportion of the dimer decreased from a control level of 43.1 $\pm$ 4.3% to a final level of 12.6 $\pm$ 3.2% (Figure 5A, lanes 1 - 8; Figure 5B) The D_2 receptor TM VII control peptide had no effect on receptor dimerization (Figure 5A, compare lanes 9 and 10) similar to the results shown using membrane preparations (Figure 4B). We also noted a modest but reproducible upward shift in the apparent molecular weight of the monomer resulting in a widening of the band as the concentration of peptide was increased (Figure 5B, inset). This suggests that as proposed above the peptide forms a stable complex with the receptor monomer thus mimicking receptor-receptor interactions.

Functional consequences of receptor dimerization

The functional significance for receptor dimerization is suggested by the inhibitory action of the TM VI peptide on receptor-stimulated adenylyl cyclase activity. As shown in Figure 6A, the addition of TM VI peptide to membrane preparations at a concentration of 0.15 μg/μl significantly reduced isoproterenol-stimulated adenylyl cyclase activity ($p < 0.05$). In contrast, neither the peptide solubilization buffer (data not shown) nor control peptides (TM VI-Ala or TM VII of the D_2 dopamine receptor) had significant effects on isoproterenol-stimulated adenylyl cyclase activity.

The effect of the peptide was receptor-specific as it had no effect on either NaF-mediated or forskolin-mediated adenylyl cyclase stimulation (Figure 6B). Notably, the ligand-

independent basal adenylyl cyclase activity was slightly inhibited by the TM VI peptide suggesting that it may effect the spontaneous activity of the receptor as well. Indeed, spontaneous receptor activity is in large part responsible for the ligand-independent adenylyl cyclase activity observed in both Sf9 and mammalian cells expressing the β_2AR (Chiciac, P., *et al.*, *Mol. Pharmacol.* 45:490-499, 1994). A receptor-dependent effect is also supported by the fact that the TM VI peptide was without effect on basal cyclase activity in Sf9 cells which were infected with the wildtype baculovirus (data not shown). Also consistent with a receptor-specific action of the peptide is the observation that D_1 dopamine receptor-stimulated adenylyl cyclase activity was not significantly affected by the TM VI peptide (Figure 6C). As was the case for the inhibition of dimerization, the inhibitory action of the TM VI peptide on receptor-mediated adenylyl cyclase activity was dose-dependent (Figure 6C). It should be noted that the peptide IC_{50} values for the inhibition of dimer formation are very similar (2.14 ± 0.05 μM and 3.2 ± 0.04 μM, respectively) thus suggesting that receptor dimerization may be an important step in β_2AR-mediated signalling. Although our data suggest a role for dimerization in receptor activity, one cannot exclude the possibility that the effect of the TM VI peptide is not directly due to an effect on the monomer:dimer equilibrium. Still, these results clearly show that this domain of the receptor is important in modulating β_2AR signal transduction. Furthermore, the peptide represents a novel pharmacological tool for the study of receptor activity.

The effect of TM VI peptide on adenylyl cyclase stimulation does not result from a loss of receptor sites as neither the affinity or the maximum number of binding sites for ^{125}I CYP were affected ($K_D = 1.8 \pm 0.5 \times 10^{-10}$ and $B_{mas} = 16.5 \pm 2$ pmol/mg protein for untreated membranes compared with $K_D = 4.2 \pm 1.5 \times 10^{-10}$ and $B_{max} = 21.3 \pm 4.5$ pmol/mg protein for TM VI peptide treated membranes, n=3 for both determinations).

Effect of the TM VI peptide on GPCR in mammalian cells

In this study, β_2AR dimers were observed in CHW cells stably transfected with the receptor (Figure 8A, inset) by immunoblotting with a polyclonal anti-β_2AR antisera.

Similar to our observations in Sf9 cells, the TM VI peptide also reduced the amount of β_2AR dimer detected in membrane derived from CHW cells (Figure 8A, inset lane 2). This peptide also reduced basal and isoproterenol-stimulated adenylyl cyclase activity in these cells while leaving forskolin- and NaF-mediated stimulation unaffected (Figure 8A). Similar findings were also obtained with LTK- cells expressing as little as 200 fmol of β_2AR/mg protein (Figure 8b). These results taken together suggest a similar functional significance for β_2AR dimerization in mammalian cells as in Sf9 cells.

The results presented here demonstrate that both human β_2AR and V2 vasopressin receptors can form SDS-resistant homodimers. For the β_2AR, the relative amount of dimer can be altered by a peptide derived from TM VI and by receptor ligands suggesting that under basal conditions there appears to be a dynamic equilibrium between monomeric and dimeric species of receptors. The data also suggest that shifting the equilibrium away from the dimeric form of the receptor interferes with the ability of the β_2AR to productively interact with its signalling pathway.

Application of Assay to other GPCRs

Higher molecular weight species have been detected in both mammalian and Sf9 expression systems for many GPCRs. These include the V2 vasopressin receptor (see discussion above - this study, Figure 3), platelet activating factor receptor, metabotropic glutamate receptor, substance P receptor, neurokinin-2 receptor, the C5a anaphylaxotoxin receptor, glucagon receptor, the dopamine D_1 receptor, D_2 receptor, the $5HT_{1B}$ receptor, the M_2 muscarinic receptor and the M_3 muscarinic receptor (see Hebert, T.E *et al.*, *J. Biol. Chem.* accepted, 1996, and references therein). Thus, GPCR-peptides and peptidomimetic compounds could be designed for these receptors that would function to as demonstrated in these examples to selectively prevent or disrupt the functional aggregation of these receptors, thereby attenuating receptor activity.

From the foregoing description, one skilled in the art can easily ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof,

can make various changes and modifications of the invention to adapt it to various usages and conditions. Consequently, such changes and modifications are properly, equitably, and "intended" to be, within the full range of equivalence of the following claims.

WE CLAIM:

1. An *in vitro* method to evaluate compounds for the ability to modulate the activity of a G protein-coupled receptor comprising detecting the ability of the test compound or compounds to alter the ratio of monomeric-to-multimeric G protein-coupled receptors.

2. The method as in claim 1, wherein said G protein-coupled receptor is selected from the group of receptors consisting of acetylcholine, adenosine, corticotropin-releasing factor, cannabinoid, dopamine, histamine, neuropeptide Y, norepinephrine, epinephrine, opioids, serotonin, vasopressin, platelet activating factor, metabotropic glutamate, substance P, neurokinin-2, C5a anaphylaxotoxin, and glucagon.

3. The method as in claim 1, wherein said G protein-coupled receptor is β_2AR.

4. An *in vitro* method to evaluate compounds for the ability to modulate the activity of a G protein-coupled receptor comprising the steps of :

(a) measuring the ratio of said monomeric receptors to multimeric receptors in a sample;

(b) incubating the test compound or test compounds with an aliquot of the sample for a limited period of time;

(c) measuring the ratio of monomeric receptors to multimeric receptors in the test aliquot;

(d) determining the extent to which said test compound or compounds altered the ratio of monomeric to multimeric receptors.

5. A test kit comprising the reagents enabling the method of claim 1.

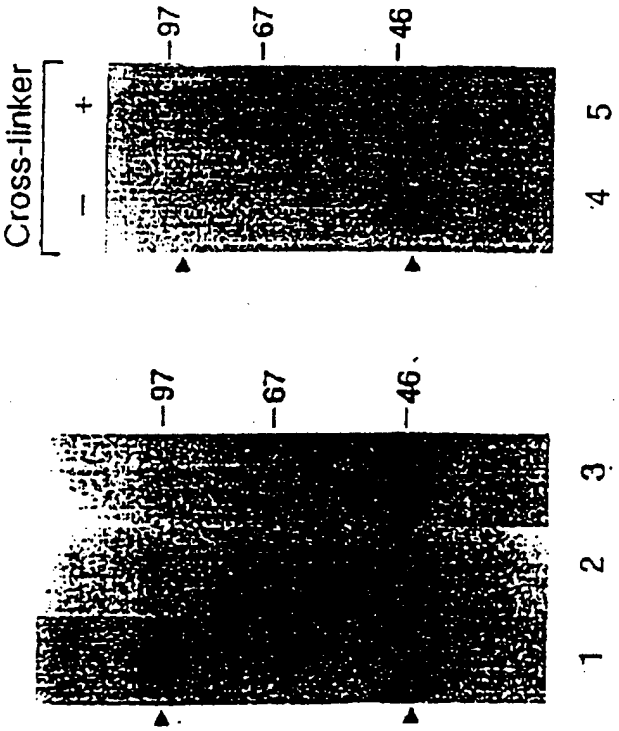


FIGURE 1

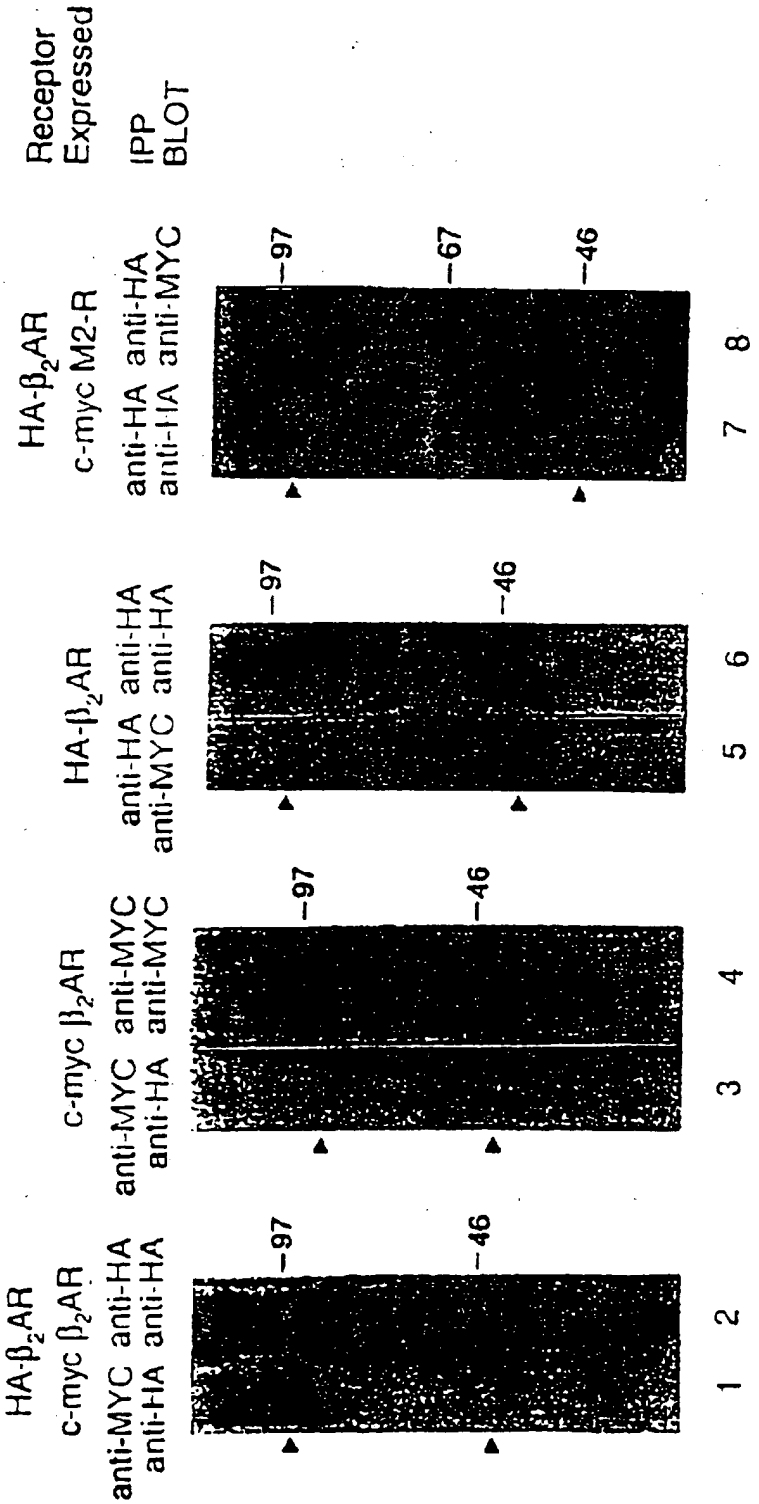

Receptor Expressed
IPP BLOT
HA-β2AR
c-myc M2-R
anti-HA anti-HA
anti-HA anti-MYC
HA-β2AR
anti-HA anti-HA
anti-MYC anti-HA
c-myc β2AR
anti-MYC anti-MYC
anti-HA anti-MYC
HA-β2AR
c-myc β2AR
anti-MYC anti-HA
anti-HA anti-HA
-97
-67
-46
1 2 3 4 5 6 7 8

FIGURE 2

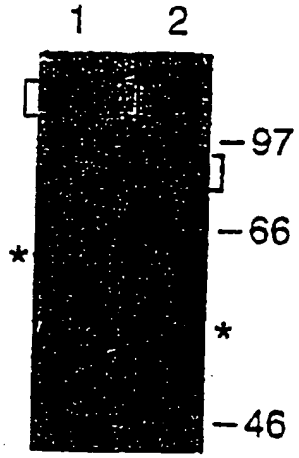


FIGURE 3

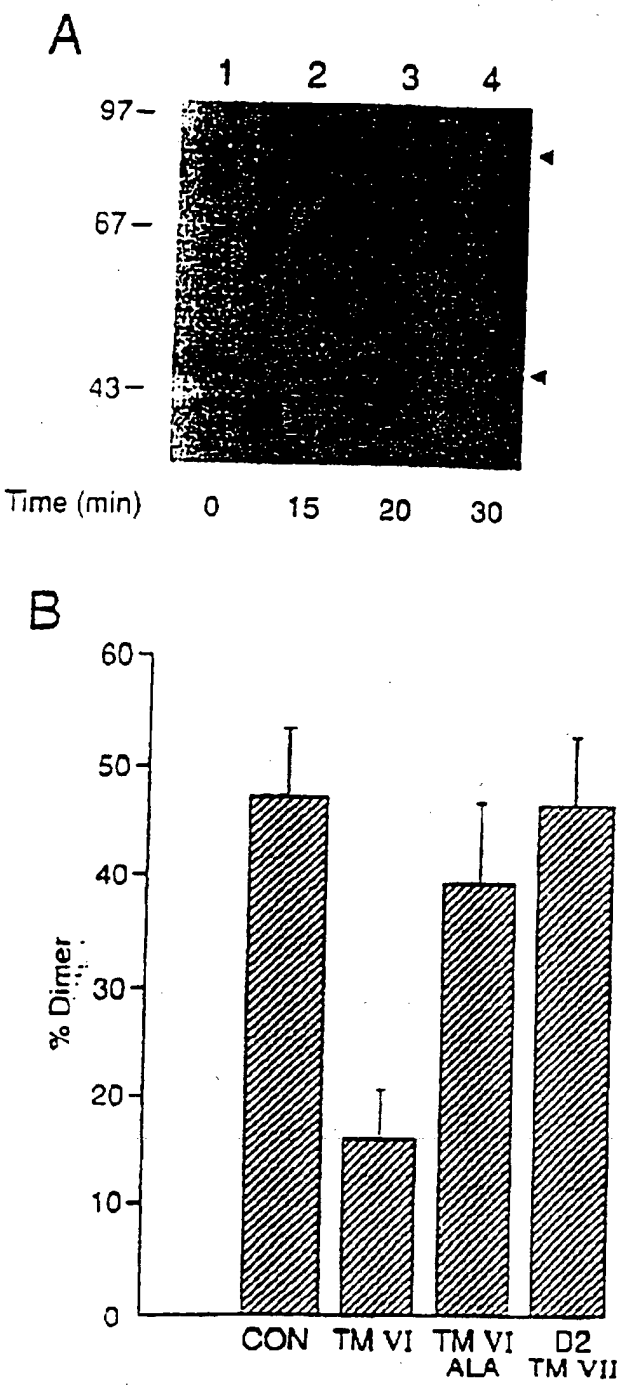

A
1
2
3
4
97–
67–
43–
Time (min)
0
15
20
30
B
60
50
40
30
20
10
0
% Dimer
CON
TM VI
TM VI ALA
D2 TM VII

FIGURE 4

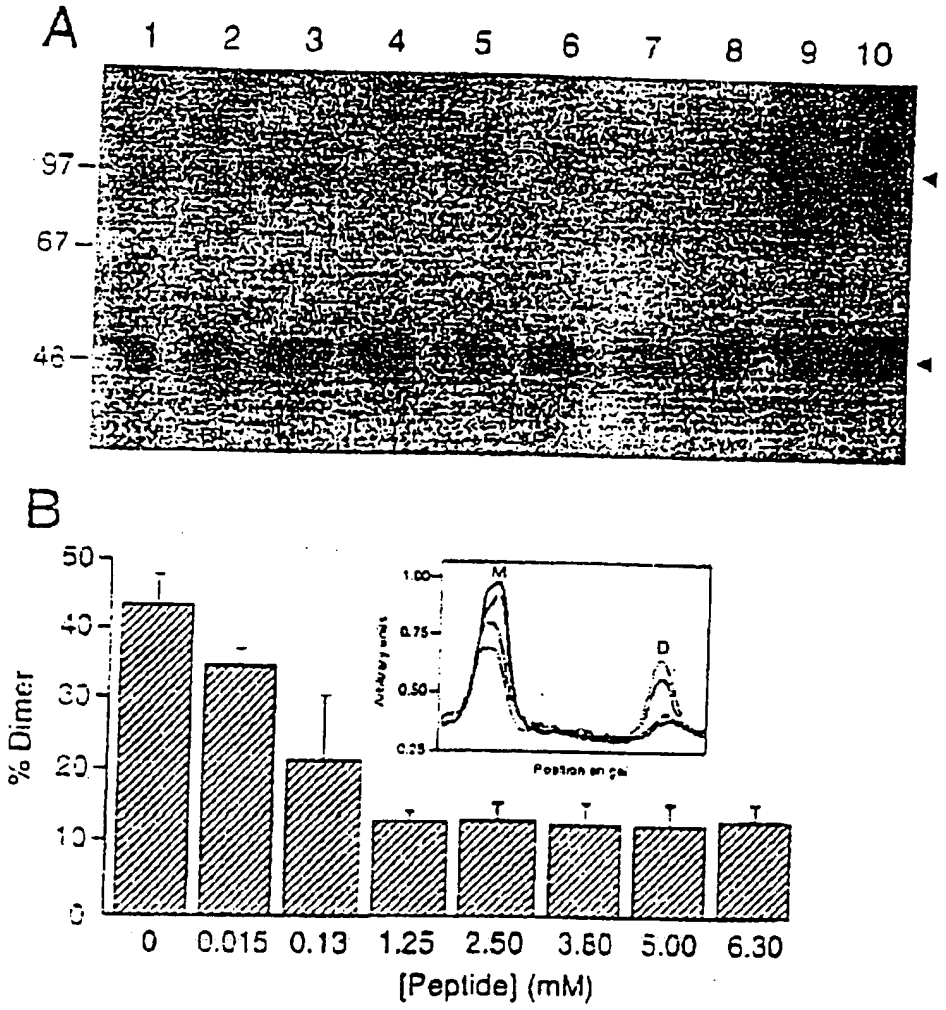

A
1 2 3 4 5 6 7 8 9 10
97
67
46
B
% Dimer
50
40
30
20
10
0
0 0.015 0.13 1.25 2.50 3.60 5.00 6.30
[Peptide] (mM)
M
D

FIGURE 5

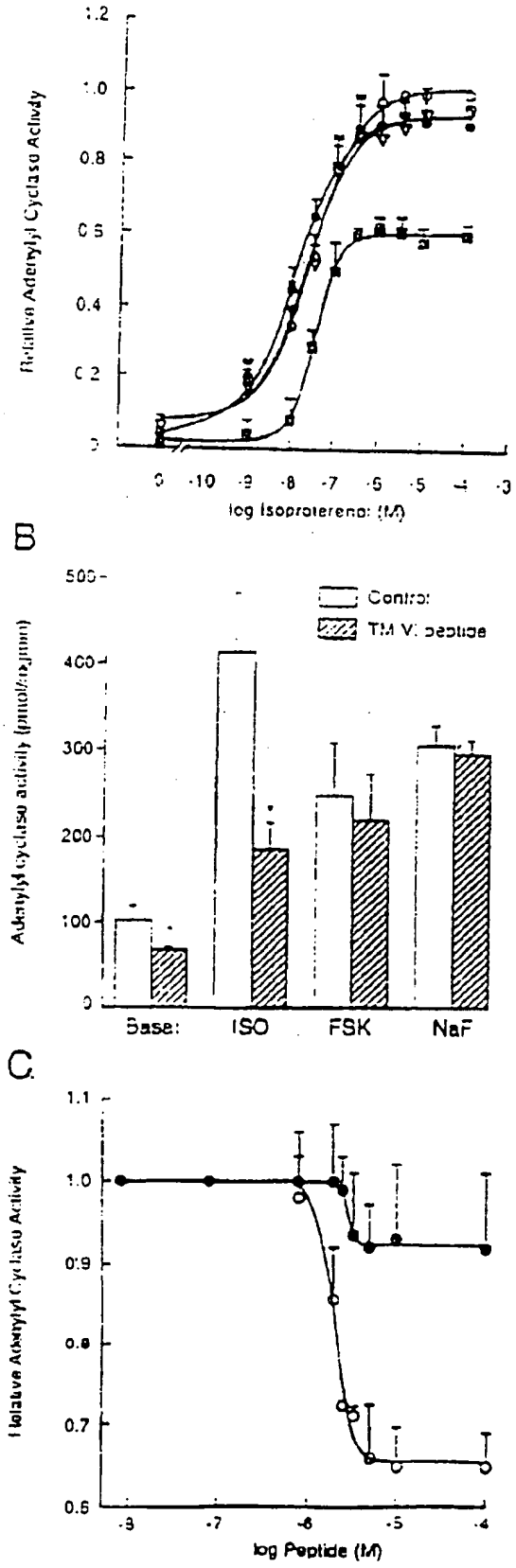
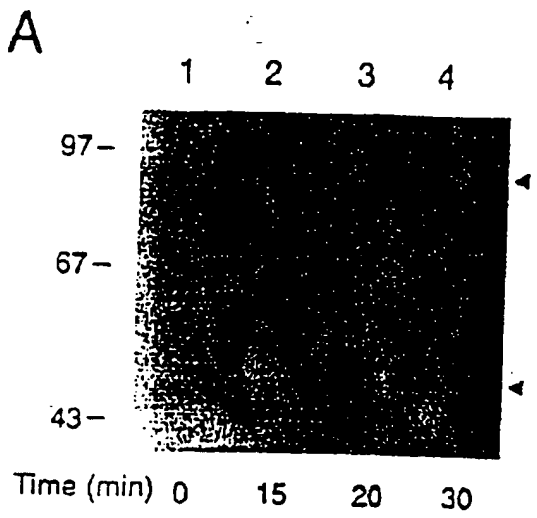

1.2
1.0
0.8
0.6
0.4
0.2
0
-10
-9
-8
-7
-6
-5
-4
-3
log Isoproterenol (M)
B
500
400
300
200
100
0
Control
TM V peptide
Base:
ISO
FSK
NaF
C
1.1
1.0
0.9
0.8
0.7
0.6
-8
-7
-6
-5
-4
log Peptide (M)

FIGURE 6


A
1
2
3
4
97–
67–
43–
Time (min) 0
15
20
30

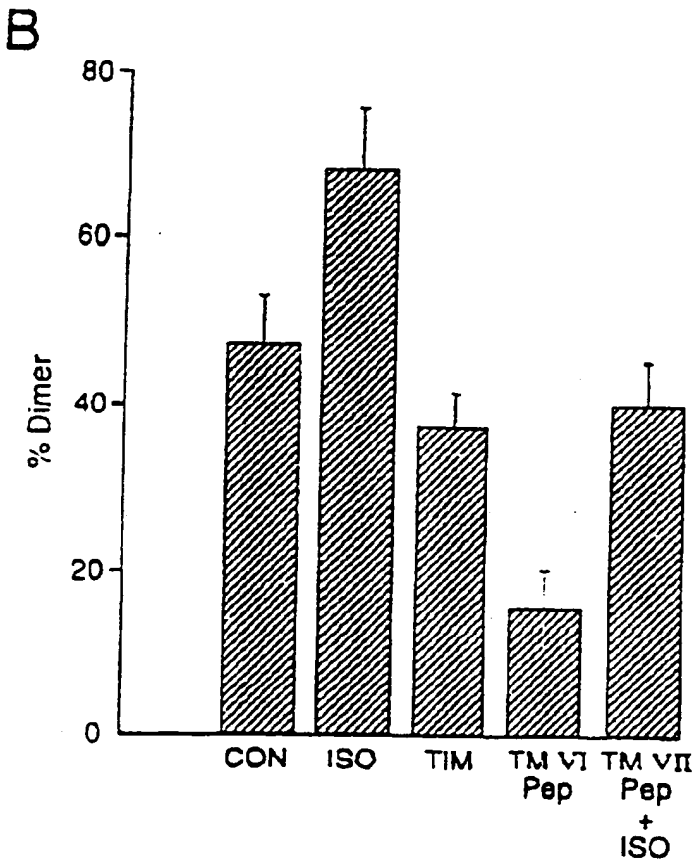

B
80
60
40
20
0
% Dimer
CON
ISO
TIM
TM VI Pep
TM VII Pep + ISO

FIGURE 7

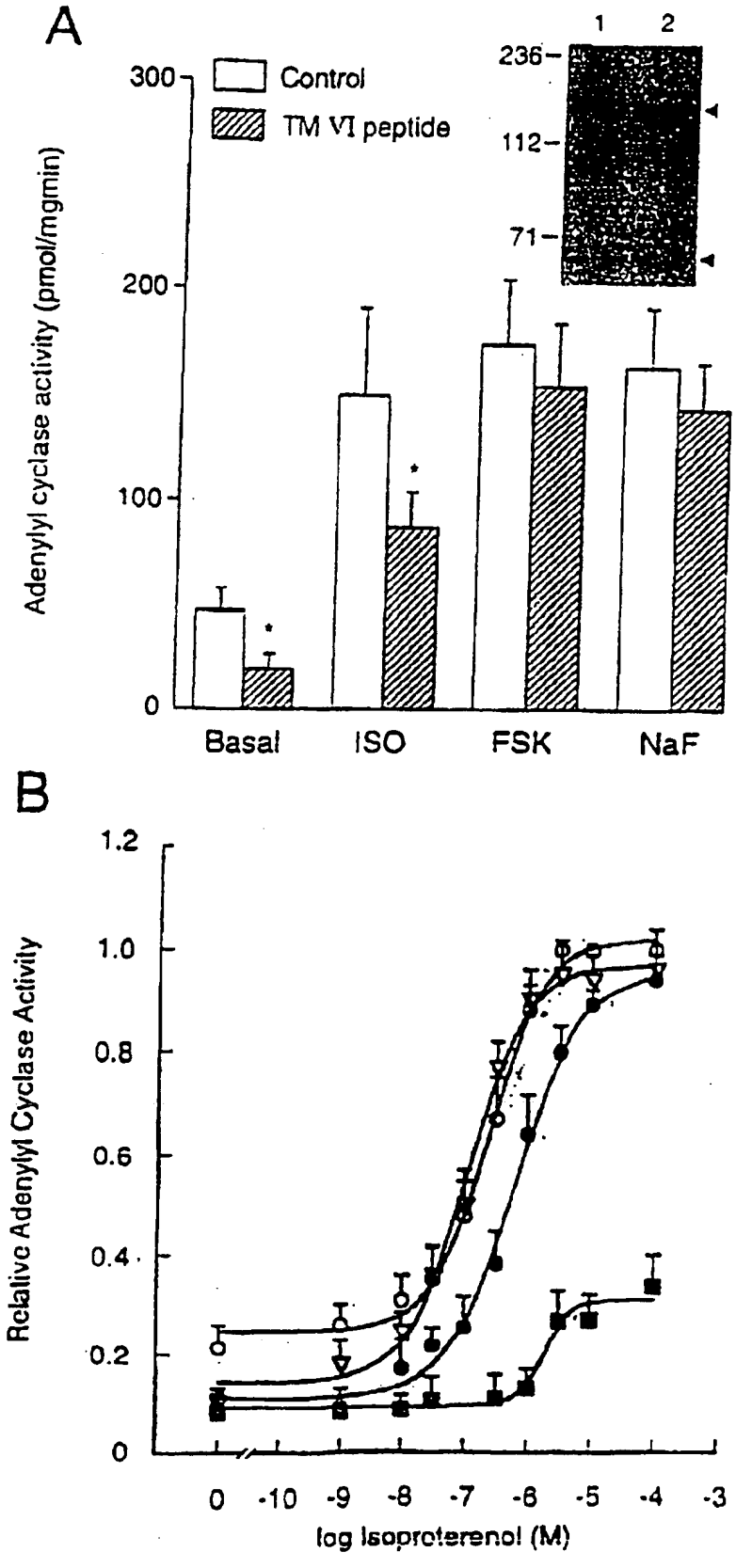

A
Control
TM VI peptide
Adenylyl cyclase activity (pmol/mgmin)
300
200
100
0
Basal
ISO
FSK
NaF
1
2
236
112
71
B
Relative Adenylyl Cyclase Activity
1.2
1.0
0.8
0.6
0.4
0.2
0
0
-10
-9
-8
-7
-6
-5
-4
-3
log Isoproterenol (M)

FIGURE 8

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(54) Title: METHOD OF ASSAYING COMPOUNDS WHICH AFFECT THE ACTIVITY OF G PROTEIN-COUPLED RECEPTORS BASED ON MEASUREMENT OF RECEPTOR OLIGOMERIZATION

(57) Abstract

This invention is a new method of assaying compounds that modulate the activity of G protein-coupled receptors based on measurement of changes in the relative proportions of monomeric to multimeric receptor polypeptides. More specifically, techniques are described herein which permit the prediction of the pharmacological efficacy of drug candidates based solely on the ability of the candidate compounds to alter the ratio of receptor monomer to homo-oligomeric forms of the receptor. This method provides a novel means of assaying compounds as potential therapeutic drugs at G protein-coupled receptors which is greatly simplified and more generally applicable than existing methods.

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INTERNATIONAL SEARCH REPORT

Internat I Application No
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A. CLASSIFICATION OF SUBJECT MATTER
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According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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A,P	WO 97 04124 A (BIOSIGNAL INCORPORATED & L'UNIVERSITÉ DE MONTREAL) 6 February 1997 see the whole document ---	1-5
T	WO 98 00538 A (BIOSIGNAL INCORPORATED & L'UNIVERSITÉ DE MONTREAL) 8 January 1998 see the whole document -----	1-5

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1

Date of the actual completion of the international search	Date of mailing of the international search report
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INTERNATIONAL SEARCH REPORT

Information on patent family members

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WO 9704124 A	06-02-97	AU 6296596 A	18-02-97
		CA 2160038 A	15-01-97
WO 9800538 A	08-01-98	NONE	